NCLX/NCKX6 is a recently identified gene responsible for a mitochondrial Na^+-Ca^{2+} exchanger (NCX_{mit}), a Ca^{2+} extrusion system from mitochondria. Although there have been accumulated data regarding roles of NCLX in various kinds of cells, little is known about the roles in cardiomyocytes, where repetitive Ca^{2+} transients occur and where a huge amount of ATP is generated and utilized. In the present study, we carried out a combination study of *NCLX* knockdown in spontaneously beating atrial cell line HL-1 and mathematical simulations. In HL-1 cells, *NCLX* knockdown by siRNA reduced the protein expression by ~50%. A cytosolic Na^+-dependent mitochondrial Ca^{2+} efflux was decelerated by knocking down *NCLX*, confirming that *NCLX* is a gene responsible for NCX_{mit} in cardiomyocytes. Interestingly, the cycle length of spontaneous Ca^{2+} oscillation and action potential generation was significantly prolonged by knocking down *NCLX*. Detailed inspection revealed that the rate of initial membrane depolarization and upstroke of Ca^{2+} rise were markedly slower in the *NCLX* knockdown cells. Furthermore Ca^{2+} content in sarcoplasmic reticulum (SR) was lower and SR Ca^{2+} reuptake was slower in the *NCLX* knockdown cells. A mathematical model of HL-1 cells showed that an automaticity of HL-1 cells are driven by a spontaneous Ca^{2+} leak from SR, called ''Ca^{2+} clock''. Analyses using the model demonstrated that blocking NCX_{mit} reduced SR Ca^{2+} content and SR Ca^{2+} leak, resulting in the prolongation of beating rate. Taken together, the NCLX has an important role in regulating cardiac automaticity by modulating ''Ca^{2+} clock''.

561-Pos Board B330

Regulation of Na^+/CA^{2+} Exchanger by Pyridine Nucleotide Redox Potential in Cardiac Myocytes

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Cardiac Na^+/Ca^{2+} exchanger (NCX) is the major Ca^{2+} efflux pathway on the sarcolemmal membrane, counterbalancing Ca^{2+} influx via L-type Ca^{2+} current during excitation-contraction coupling. In humans, guinea pigs and large mammals, NCX removes 20-25% of total cytosolic Ca^{2+} during relaxation. Therefore, altered NCX activity can modulate SR Ca^{2+} load and contribute to abnormal Ca^{2+} handling and arrhythmias. Here, we demonstrate that: 1) increased NADH inhibits NCX current (I_{NCX}); 2) NCX inhibition by NADH is reactive oxygen species (ROS)-dependent; 3) the NADH-induced ROS is independent of the mitochondrial electron transportation chain (ETC). I_{NCX} was measured with whole cell patch-clamp at 37°C after dialyzing the cell with various pipette solutions. With 400μM NADH in the pipette solution, I_{NCX} (at +80mV) was reduced by 65% compared to control. Endogenously elevated cytosolic NADH by lactate perfusion (1mM) also inhibited I_{NCX} by 79% (at +80mV) compared to control without lactate perfusion. Measurement of ROS revealed that increasing cytosolic NADH increased ROS accumulation. The NADH-induced increase in ROS, as well as the inhibition of I_{NCX}, was abrogated by co-dialysis with the H_2O_2 scavenger catalase, demonstrating that NCX inhibition is mediated by ROS. Inhibition of ETC with rotenone did not reverse NCX inhibition by NADH, whereas D diphenylene iodonium (DPI), which inhibits both the ETC and NAD(P)H oxidases, abolished NCX inhibition. Lactate also induced ROS production in cell suspensions in a dose-dependent manner, which was inhibited by DPI but not by rotenone, NaCN, or antimycin A. Our findings indicate that NCX is regulated by cytosolic NADH/NAD^+ redox status through a ROS-dependent mechanism involving NAD(P)H oxidase, a mechanism that is likely to have profound effects on Ca^{2+} homeostasis under conditions that alter cytosolic pyridine nucleotide redox state, for example, during ischemia-reperfusion, diabetes or chronic heart failure.

562-Pos Board B331

Is ASP-454 Necessary for Na^+ and K^+ Binding in the Glutamate Transporter EAAC1?

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The glutamate transporter Excitatory Amino Acid Carrier 1 (EAAC1) catalyzes rapid removal of glutamate, the major excitatory neurotransmitter in the brain, from the extracellular space, preventing its accumulation to neurotoxic levels. Uphill substrate transport is coupled to co-transport of 3 Na^+, 1 H^+, and counter-transport of 1 K^+ ion. The cation binding sites are yet to be fully identified in this transporter. Here, we investigate the role of the highly conserved D454 residue which is thought to contribute its side chain to coordinating these cations. D454 was mutated to the non-ionizable asparagine, as well as alanine. K^+-induced relocation was impaired in EAAC1-D454N, as the transporter no longer catalyzed forward transport. Our findings indicated that K^+ could still bind to EAAC1-D454N, but with reduced apparent affinity. Na^+/glutamate exchange was functional in EAAC1-D454N, as demonstrated by the presence of transient currents following rapid glutamate application and voltage jumps. In the D454A mutant, glutamate application inhibited inward leak anion currents, and transient currents in response to voltage jumps were abolished. This is consistent with Na+ binding being impaired in this transporter. K^+ binding was also eliminated by the D454A mutation. Although D454 is proposed to participate in Na+ binding, its protonation state is yet to be established. pKa calculations for the bacterial homologue Gltph indicate that D405 (analogous to D454) is protonated at physiological pH with a highly perturbed pKa in the range of 7.6 to >14. Valences calculated using the Poison-Boltzmann equation for EAAC1 with D454 protonated (0.45) were close in value to the experimental values obtained for EAAC1-D54N (0.71) and EAAC1-WT (0.64). Together, our results suggest that D454 is protonated under physiological conditions, and participates in Na^+ as well as K^+ binding.

563-Pos Board B332

Transition Metal FRET to Study Extracelluar Gate Movement in Glutamate Transporter

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In the crystal structure of the bacterial glutamate transporter homologue GltPH, an L-aspartate and two Na^+ ions are bound within the core of each subunit and bordered by two hairpin loops: HP1 from the intracellular side and HP2 from the extracellular side. Notably, in another crystal structure of GltPH bound with L-threo-β-benzyloxyaspartate (L-TBOA), a non-transportable substrate analogue, TBOA locks the transporter in an outward-facing conformation similar to the aspartate-bound complex, except that HP2 adopts an ''open'' conformation, exposing the substrate binding site to the extracellular solution. This suggests that HP2 may serve as an extracellular gate. In addition, our published voltage clamp fluorometry measurements show that there are significant Na^+-dependent movements of HP2 preceding glutamate binding. Here we use transition-metal FRET to study the movements of HP2 in human glutamate transporter EAAT3 upon binding of substrate and inhibitor. Compared to the classical FRET methods which have previously been used by our lab to measure conformational changes in EAAT3 during the glutamate transport cycle, the transition metal ion FRET pairs are smaller in size and have a shorter R0 value than the traditional FRET pairs, which enables us to measure distance change within a very short range to better study the conformation change of HP2 during ion and substrate binding. By labeling the FRET donor fluorophore and the transition metal ion acceptor at different positions of glutamate transporter EAAT3, we measured intrasubunit distance changes of the HP2 region in the presence of glutamate compared with in the presence of L-TBOA.

564-Pos Board B333

Insights into the Human Glutamate Transporters from the Bacterial Homolog Glt(Ph)

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Human glutamate/aspartate transporters cotransport three Na^+ and one H^+ ions with the substrate and countertransport one K^+ ion. The archeal homolog GltPh, which has a 36% sequence identity with the human transporters, transports aspartate and three sodium ions across the lipid membrane. We have previously performed Free Energy Perturbation (FEP) simulations of ligand binding to GltPh in the extracellular(EC)-facing state, showing that the substrate binds after two Na^+ ions, with a strong water-mediated coupling between the second sodium ion to bind (Na1) and the substrate. We have also performed simulations and mutagenesis experiments to locate the binding site of the third sodium ion (Na^3), which could not be resolved in the crystal structure. More recently, we have turned our focus to the intracellular(IC)-facing conformation of GltPh. By running long unbiased simulations with different ligands bound to the transporter, we were able to describe in details the gating mechanism and point out the differences to the EC-facing state. The IC-facing FEP results demonstrate that the unbinding of the ligands happens in a symmetrical manner, with the ligands being released in the reverse order of their binding. The Na^3 ion is, therefore, the first ligand to bind and the last to unbind. By means of free energy calculations, Kramers' theory and experiments, we are able to point out the unbinding of the Na^3 ion as the rate-limiting step in the transport mechanism. With all this information, we have built a homology model for a human glutamate transporter (EAAT1) in different conformations, which can reproduce some of the differences observed in the transport mechanism of the human and the bacterial transporters.